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13. ABSTRACT ***(Maximum 200 Words)***
The search of chemopreventive strategies for breast cancer is imperative. It is vital to identify critical early events that can increase the risk that a normal epithelial mammary cell may be transformed into a breast cancer cell. Exposure to estrogen, already, recognized as a predisposing event for breast cancer, formed the rationale for preventive trials based on an antiestrogen compound (tamoxifen). This proposal explores the mechanism of another likely predisposing event for breast cancer, the loss of expression of the retinoic acid receptor beta (RAR beta), a nuclear receptor that responds to derivatives of vitamin A. Loss of expression of RAR beta was reported in breast cancer, as well as in other epithelial cancers. Our IDEA project aims to understand the mechanisms involved in RAR beta loss, in order to devise strategies not only to reverse this loss, but also to prevent it. In the first year of investigation, we found that a mechanism of transcriptional silencing of genes called hypermethylation is an important factor of irreversible RAR beta loss in breast cancer cells. Moreover, we found that relieving chromatin repression, by either demethylation or reacetylation at RAR beta promoter level, can restore RAR beta expression. Now, we plan to test whether loss of RAR beta expression can be prevented in breast cells by maintaining an appropriate activity of the RAR beta P2 promoter to deter the occurrence of epigenetic events that now we know are responsible for RAR beta silencing and RA-resistance.

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RAR BETA METHYLATION AND LOSS OF RAR BETA EXPRESSION IN BREAST CANCER (PI : SACCHI N)

INTRODUCTION

This proposal explores the mechanism of another likely predisposing event for breast cancer, the loss of expression of the retinoic acid receptor beta (RAR beta), a nuclear receptor that responds to derivatives of vitamin A, including retinoic acid (RA) (Chambon, 1996). Loss of expression of RAR beta was reported in both breast cancer cell lines and primary breast tumors (Swisshelm et al., 1994; Seewaldt et al., 1995; Li et al., 1995; Tsou et al., 1998; Widshwendtner et al., 1997; Xu et al., 1997). The expression can be reinduced in some breast cancer lines by RA treatment, whereas in other breast cancer cell lines RAR beta expression cannot be reversed (Liu et al., 1997). These breast cancer cell lines can be considered a model of primary breast tumors resistant to RA-differentiation therapy (Baust et al., 1996).
Our project aims to understand the causes of RAR beta loss and devise strategies both to revert this loss and possibly to prevent it. We originally proposed to investigate:
1) whether a mechanism of transcriptional silencing of genes called hypermethylation is a factor of the irreversible RAR beta loss in breast cancer (Task 1);
2) whether RAR beta expression can be restored by overriding methylation either altering the chromatin state or by demethylating the RAR beta 2 promoter (Task 1);
3) whether methylation of RAR beta can be prevented by preventing the inactivity of RAR beta 2 promoter (Task 2).
We report that indeed hypermethylation is a factor in RAR beta loss and that RAR beta loss can be restored by chromatin remodeling drugs. The proposed course aims at understanding how hypermethylation can be prevented, in order to prevent RAR beta loss and its consequences.

BODY

In the approved Statement of Work (SOW) we originally proposed to perform two Tasks, 1 and 2.

Task 1. To determine the expression of the RAR beta gene and the methylation status of the RAR beta 2 promoter region in human epithelial mammary cells (HMEC) and breast cancer cell lines and analyze the demethylating effect of 5-Aza-2'-deoxycytidine (5 -Aza-CdR) on re- activation of RAR beta (months 1-12).

We were able to successfully complete all the experiments planned to accomplish Task 1. First, we tested the hypothesis that loss of RAR beta activity occurs as a result of multiple factors, including aberrant methylation of the regulatory region of RAR beta, an epigenetic change which can pattern the RAR beta chromatin state and influence gene activity. Using methylation-specific PCR (MSP), we found hypermethylation in the promoter P2 of the RAR beta gene in a significant proportion of both breast cancer cell lines and primary breast tumors (See Appendix 1, paper by Sirchia et al., Oncogene, 2000).
Second, we observed that the chromatin changes induced by methylation at the level of RAR beta promoter can be reversed, with subsequent reexpression of the gene, and

restoration of RA sensitivity. We showed that treatment of breast cancer cell lines with a methylated P2 promoter, by means of the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (5-Aza-CdR), led to demethylation of the RAR beta 2 promoter and expression of RAR beta indicating that DNA methylation is at least one factor, contributing to RAR beta inactivity (see Appendix 1, Sirchia et al., Oncogene, 2000). We now know, that DNA methylation causes an altered acetylation by recruiting the MeCP2/Sin3A/HDAC complexes (Nan et al., 1998; Wade et al., 1998). For this reason, we tested whether RAR beta activity could be restored by simply altering the repressive chromatin state at the level of RAR beta 2 promoter. We found that the more stable repressive RAR beta 2 status in the RA-resistant MDA-MB-231 cell line can be alleviated by the HDAC inhibitor, trichostatin A (TSA), with restoration of RA-induced RAR beta transcription. In Appendix 1, we enclose two manuscripts (Sirchia et al., 2000; Virmani et al., 2000) and three abstracts summarizing the results of: a) our breast cancer study and b) a collaborative study on lung cancer with Dr. A. Virmani at the Hamon Center for Therapeutic Oncology Research in Dallas, TX.
Dr. Virmani extended the experimental approach devised for our breast cancer study to demonstrate that RAR beta hypermethylation results in RAR beta loss in a high proportion of breast cancers. Altogether these results show that hypermethylation associated with RAR beta loss is not limited to breast cancer alone and broaden the scope of our preventive study.

Hypermethylation of RAR beta gene in breast cancer may be utilized for several clinical applications including:
1) *Early diagnosis of breast cancer*. Detection of methylation by methylation specific PCR (MSP) (Herman et al., 1996) is very sensitive. Each tumor type, including breast cancer has specific methylated genes that can be exploited as diagnostic and early detection markers (Belinski et al., 1998). A methylated gene in order to be useful as an early detection marker must fulfil a number of criteria, including: a) to be present in at least 30% of breast cancers; b) to be unmethylated in peripheral blood lymphocytes and normal stroma, as well as in stroma and support tissue adjacent to the breast tumor; c) should be methylated in early disease stage. Each of these criteria is met by RAR beta that can be included in a panel of hypermethylated markers for breast cancer detection (Belinski et al, 1998). Currently, a panel of hypermethylated markers including RAR beta is used at JHU for early detection of tumor cells on breast ductal cell samples obtained by the procedure of ductal lavage technique.

2) *Detection of RA-resistant tumors*. It has been reported that a fraction of breast cancers are refractory to RA-differentiation therapy (Baust et al., 1996). Now we know that breast cancer with hypermethylated RAR beta are RA-resistant. Detection of the fraction of breast cancer that are methylated can identify the breast cancers that are unlikely to respond to RA-differentiation therapy. This may assist in the selection of patients that may benefit from therapeutic protocols including RA.

3) *Improving the treatment of RA-resistant breast cancer*. We have shown that by using RA in combination with chromatin-remodeling drugs, like the histone deacetylase inhibitor Trichostatin A, is possible to restore RA-sensitivity in breast cancer cells in

vitro. This suggests that treatment of RA-resistant tumors might be improved by combining RA to histone deacetylase inhibitors. This drug combination has already been used in the treatment of leukemia (Warrell et al., 1998) and may be exploited to overcome the hurdle of RA-resistance in breast cancer.

Task2. To attempt the induction of the methylation status of the RAR beta P2 promoter by inhibiting RAR beta activity (months 13-24)

Breast cancer could be prevented if we could identify ways to prevent the occurrence of key molecular changes leading to transformation of mammary epithelial cells. Loss of RAR beta seems to be a key change. Recently, it has been discovered that loss of RAR beta causes the down regulation of expression of the intracellular adhesion molecule-1 gene (ICAM) and the MHC class I heavy chain . This fact strongly indicates that RAR beta deficient tumor cells may escape immunological surveillance (Toulouse et al., 2000). In light of this finding, it is clear that prevention of irreversible RAR beta gene inactivity may be very relevant.

We would like to prevent RAR beta loss by acting on the factors responsible for RAR beta gene inactivity. We hypothesize that DNA-methylation is secondary to prolonged inactivity of the RAR beta P2 promoter. RAR beta P2 is regulated in the presence of RA by its receptors RAR alpha and RAR beta itself (Gudas et al., 1994; Chambon, 1996). In the presence of RA the receptors activate RAR beta P2 by tethering protein complexes with histone acetylase activity (HAT) and, in the absence of RA, of protein complexes with histone deacetylase activity (HDAC) (Chambon, 1996). Histone deacetylation of P2 may predispose to DNA methylation, a condition that might further attract histone deacetylation mediated by the MeCP2/Sin3A/HDAC corepressor complex (Nan et al., 1998; Wade et al., 1998; Razin, 1998; Ng and Bird, 1999; Jones and Wolffe, 1999). The idea that gene inactivity invites"de novo methylation was first proposed by Bird, back in 1986. This suggestion was further refined after the discovery of the mechanistic link between DNA methylation and chromatin conformation. Ng and Bird recently proposed that: "DNA methyltransferase –either independently or assisted by accessory proteins – may be capable of reading the histone acetylation pattern on the chromatin and its de novo methyltransferase activity can respond differentially to different states of chromatin modification. In this case, deacetylated chromatin would provoke de novo methylation. This self-reinforcing mechanism, supported by DNA methylation and histone deacetylation, could provide a stable state of inactive chromatin, unless overcome by other mechanisms".

The RAR beta P2 promoter can provide an ideal system to test this as well as our hypothesis.

Methods

We will culture unmethylated HMEC or unmethylated breast cancer cell lines (Hs578t and T47D) in the presence of LE135, an antagonist of RAR beta kindly provided by Dr. Hashimoto (Tokyo, Japan) in the presence and absence of RA. This culture conditions should force the P2 promoter to inactivity. Cells will be tested for RAR beta loss (by means of RT-PCR). In parallel we will be testing for changes in the histone acetylation status of the chromatin embedding the RAR beta promoter 2 (by Chromatin Immunoprecipitation analysis) and hypermethylation of RAR beta (by MSP analysis). ChIP and MSP analyses will be performed as previously described (Keshet ET al.,

1986; Hebbes ET al., 1994; Eden ET al., 1998).
The approach proposed to perform this Task 2 is similar to the original one. To better address the research topic, we propose to monitor in addition to DNA hypermethylation also the acetylation status of P2. We have already successfully set up the ChIP assay to test the acetylation of histone H3 and H4 in cells with both an unmethylated (Hs578t and T47D) and a methylated promoter (MCF7 and MDA-MB-231).

Expected Outcome: A direct correlation between induction of RAR beta inactivity and occurrence of changes in the acetylation/hypermethylation at RAR beta P2 will indicate that we can prevent the occurrence of RAR beta loss by devising strategies to prevent the RAR beta P2 inactivity. These strategies may be the focus of future investigations. One strategy could be to maintain supraphysiological concentration of RA in epithelial cells (Minna and Mangeldorf, 1997). RA has been already explored as a chemoprevention agent. However, despite the potential, the use of RA has been limited in the chemopreventive setting by its toxicity. Several new retinoids are being synthesized and tested in vitro and in preclinical studies. Another strategy would be to increase the levels of endogenous RA by inhibiting the cytochrome P450 –mediated catabolism of RA using a novel class of agents, the retinoic acid metabolism blocking agents (RAMBAs). Such as Liarazole, already used in clinical trials (Miller, 1998).

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that the RAR beta promoter 2 gene is hypermethylated in breast cancer cell lines and approximately one third of primary breast cancers
- Evidence that hypermethylated RAR beta meets the criteria for an early detection methylation marker and can then be used in combination with other methylated markers for molecular analysis of ductal breast cancer cells of samples like nipple aspirates and ductal lavage fluids
- Evidence that methylation is at least one factor in silencing RAR beta gene expression
- Evidence that RAR beta expression can be restored by the demethylating agent 5-Aza-CdR
- Evidence that RAR beta expression can be restored by combining the histone deacetylase inhibitor trichostatin A (TSA) and RA
- Evidence that reacetylation of the promoter containing the RAR beta promoter is necessary and sufficient to restore expression even from a methylated RAR beta 2 promoter

REPORTABLE OUTCOMES

Two Manuscripts

1) Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S, Sacchi N Evidence of epigenetic changes affecting the Chromatin State of the retinoic acid receptor beta 2 promoter in breast cancer cells. Oncogene, 19, 1556-1563, 2000

2) Virmani AK, Rathi A, Zochbauer-Muller S, Sacchi N, Fukuyama Y, Bryant D, Maitra A, Heda S, Fong KM, Thunnissen F, Minna JD, Gazdar AF Promoter methylation and silencing of the retinoic acid receptor –beta gene in lung carcinomas. JNCI 92, 1303-1307, 2000

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Silencing of the retinoic acid receptor beta (RAR beta) by hypermethylation in human breast cancer49th ASHG Meeting, October 1999

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3) Sirchia S, Subramanyan S, Sironi E, Sukumar S, Sacchi N
DNA methylation and chromatin state of the retinoic acid receptor beta (RAR beta)promoter in breast cancer cells. Cancer Genetics and Tumor Suppressor Genes Meeting, Cold Spring Harbor, August 2000

One Presentation

1) N Sacchi "Causes and consequences of chromatin remodeling in cancer cells". Special Conference Greenbaum Cancer Center, University of Maryland Cancer Center, June 15, 2000 (Hosts: Dr. Sandford Stass and Dr. Judith Karp)

CONCLUSIONS

At this time we provide evidence that DNA-methylation at RAR beta P2 promoter in breast cancer cells is an important factor affecting RAR beta transcription.
We obtained preliminary evidence that demethylation and histone acetylation of RAR beta P2 promoter are related, but distinct mechanisms for destabilizing chromatin and reactivating transcription in methylated cell lines.
We propose to continue this study to analyze whether prolonged (forced) inactivity of RAR beta is a prerequisite for the occurrence of hypermethylation/deacetylation of the P2 promoter and irreversible RAR beta loss.
This work has diagnostic, therapeutic and preventive implications.
Diagnostic implications: Knowledge of RAR beta P2 methylation state of primary breast cancers is useful to identify tumors that are likely to respond to RA-differentiation therapy. Moreover, hypermethylated RAR beta can be used in combination with other hypermethylated markers for early detection of breast cancer on ductal breast cells obtained with procedures such as ductal lavage and nipple aspiration.
Therapeutic implications: The possibility to re-induce RAR beta activity in RA-resistant breast cancer cells, using both TSA and RA, a combination proven to be effective for treating leukemia (Grignani et al., 1998; Guidez et al., 1998; He et al., 1998; Lin et al., 1998; Warrell et al, 1998), might have implications also in the treatment of RA-resistant

breast tumors.
Preventive implications: DNA-methylation might be secondary to the inactive state of the RAR beta P2 promoter and might contribute to the extinction of RAR beta transcription. If we will prove that this hypothesis is true, RAR beta loss might be prevented in the presence either of supraphysiological levels of RA or other synthetic retinoids, or by retinoic acid metabolism blocking agents (RAMBAs) such as Liarazole (Miller, 1998).

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APPENDIX 1

Task 1

Copies of the following papers

1) Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S, Sacchi N Evidence of epigenetic changes affecting the Chromatin State of the retinoic acid receptor

beta 2 promoter in breast cancer cells. Oncogene, 19, 1556-1563, 2000

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Oncogene (2000) 19, 1556–1563

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Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor β2 promoter in breast cancer cells

Silvia M Sirchia[1,2], Anne T Ferguson[3,5], Elena Sironi[2], Smitha Subramanyan[3], Rosaria Orlandi[4], Saraswati Sukumar[3] and Nicoletta Sacchi*[,2]

[1]*Laboratory of Human Genetics, Hospital San Paolo, University of Milan, Milan, Italy;* [2]*Department of Biology, University of Milan, Milan, Italy;* [3]*Breast Cancer Program, Oncology Center, Johns Hopkins University, Baltimore, Maryland, USA;* [4]*Instituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy*

Retinoic acid (RA)-resistance in breast cancer cells has been associated with irreversible loss of retinoic acid receptor β, *RARβ*, gene expression. Search of the causes affecting *RARβ* gene activity has been oriented at identifying possible differences either at the level of one of the *RARβ* promoters, *RARβ2*, or at regulatory factors. We hypothesized that loss of *RARβ2* activity occurs as a result of multiple factors, including epigenetic modifications, which can pattern *RARβ2* chromatin state. Using methylation-specific PCR, we found hypermethylation at *RARβ2* in a significant proportion of both breast cancer cell lines and primary breast tumors. Treatment of cells with a methylated *RARβ2* promoter, by means of the DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (5-Aza-CdR), led to demethylation within *RARβ2* and expression of *RARβ* indicating that DNA methylation is at least one factor, contributing to *RARβ* inactivity. However, identically methylated promoters can differentially respond to RA, suggesting that *RARβ2* activity may be associated to different repressive chromatin states. This supposition is supported by the finding that the more stable repressive *RARβ2* state in the RA-resistant MDA-MB-231 cell line can be alleviated by the HDAC inhibitor, trichostatin A (TSA), with restoration of RA-induced *RARβ* transcription. Thus, chromatin-remodeling drugs might provide a strategy to restore *RARβ* activity, and help to overcome the hurdle of RA-resistance in breast cancer. *Oncogene* (2000) **19,** 1556–1563.

Keywords: breast cancer; DNA methylation; chromatin remodeling; retinoic acid receptor (RAR)β

Introduction

Retinoic acid (RA) controls fundamental developmental processes, induces terminal differentiation of myeloid progenitors and suppresses cancer and cell growth (Smith *et al.*, 1992; Gudas *et al.*, 1994). RA activity is mediated by nuclear receptors, the retinoic acid receptors, RARs, that act as RA-dependent transcriptional activators in their heterodimeric forms with retinoid X receptors, RXRs (Chambon, 1996). RARs induce local chromatin changes at level of target genes, containing responsive RA elements (RAREs) by recruiting multiprotein complexes with histone acetyltransferase (HAT) activity and histone deacetylase (HDAC) activity, that dynamically pattern chromatin modification and regulate gene expression (see for review Chambon, 1996; Minucci and Pelicci, 1999).

RARs and RXRs, when disrupted, result in severe developmental defects and neoplastic transformation (Smith *et al.*, 1992; Gudas *et al.*, 1994; Chambon, 1996). In breast cancer cells, the expression of one member of the *RAR*s family, *RARβ* is found consistently downregulated or lost (Roman *et al.*, 1992; Shao *et al.*, 1994; Swisshelm *et al.*, 1994; Li *et al.*, 1995; Widshwendtner *et al.*, 1997; Xu *et al.*, 1997; Liu *et al.*, 1997). *RARβ* downregulation can be reversed by RA in estrogen receptor (ER)-positive, but not in ER-negative breast carcinoma cell lines, believed to represent more advanced forms of tumors (Liu *et al.*, 1997). Loss of RA-induced *RARβ* expression is considered a crucial step in the development of RA-resistance in breast carcinogenesis. A complex regulatory region, with two promoters regulates *RARβ* gene expression. Only one promoter, *RARβ2*, containing several RA-response elements, including a canonical and an auxiliary RA response element, βRARE (de The' *et al.*, 1990; Valcarel *et al.*, 1994) is active in human mammary epithelial cells (HMEC). The transcription of the *RARβ2* promoter is mediated by multiple RARs including, RARα and RARβ itself (Chiba *et al.*, 1997) able to recruit coactivator and corepressor protein complexes with HAT/HDAC activities, respectively (Chambon, 1996). To understand why *RARβ* activity is downregulated, or lost, in breast cancer, intense search has been oriented at identifying possible alterations affecting either the *RARβ2* promoter, or regulatory factors (Seewaldt *et al.*, 1995; Widschwendtner *et al.*, 1997; Xu *et al.*, 1997; Tsou *et al.*, 1998; Folkers *et al.*, 1998).

DNA methylation is an epigenetic change that induces chromatin modifications and repression of transcription via a methyl CpG binding protein MeCP2, and recruitment of a Sin3A/HDAC corepressor complex (Nan *et al.*, 1998; Wade *et al.*, 1998; Razin, 1998; Ng and Bird, 1999; Jones and Wolffe, 1999). For this reason, we decided to investigate whether *RARβ2* promoter was affected by DNA methylation. Indeed, we found hypermethylation at the *RARβ2* promoter both in breast carcinoma cell lines, and a significant proportion of primary breast tumors. Treatment with the methyltransferase inhibitor

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5-Aza-CdR partially reversed the DNA methylation state, and restored *RARβ* transcription, thus indicating that DNA methylation is at least one factor contributing to *RARβ* inactivity. However, the available data indicate that DNA methylation is only a component of the observed *RARβ* gene inactivity. Very likely, RA-inducibility of *RARβ* gene is influenced by modifications altering *RARβ2* chromatin, produced by the nuclear receptors that act at βRARE (*RARα* and the same *RARβ*), as well as DNA methylation.

Results

The RARβ2 *promoter is methylated in breast cancer cell lines independently of their ER status and RA-inducibility*

RARβ transcription was first tested in a panel of breast cancer cell lines grown in the absence of exogenous RA, by reverse transcriptase-PCR (RT–PCR), using primers encompassing exons 5 and 6 (de The' *et al*., 1990; van der Leede *et al*., 1992; Toulouse *et al*., 1997). Under these conditions, only one cell line, Hs578t, produced a detectable 256 bp RT–PCR product (Figure 4a). Thus, we confirmed previous reports that *RARβ* gene expression is down regulated/lost in breast cancer cell lines. Growing cells in the presence of RA can assess the distinction between downregulation and loss. As previously reported (Swisshelm *et al*., 1994; Liu *et al*., 1997; Shang *et al*., 1999), we observed induction of *RARβ* expression and growth inhibition in T47D, MDA-MB-435, MCF7 and ZR75-1 cell lines treated for 48 h with 1 μM RA, but not in the MDA-MB-231 and MDA-MB-468 cell lines.

To see whether the *RARβ2* methylation status correlated with the ER status, we examined the methylation status at *RARβ2* in a panel of ER-positive (MCF7, T47D, ZR75-1) and ER-negative (Hs578t, MDA-MB-231, MDA-MB-435, MDA-MB-468) cell lines.

By Southern blotting we analysed the CpG island of the *RARβ2* promoter within a 7.5 kb *Xba*I DNA fragment encompassing the TATA box, the βRARE, the transcriptional start site (TS) and the 5′ untranslated region of exon 5 (Figure 1a). In this region we can identify nine *Hpa*II sites (Shen *et al*., 1991; Baust *et al*., 1996). The DNA methylation status was analysed by using the methylation-sensitive enzyme, *Hpa*II (Figure 1b). *Msp*I, the isoschizomer of *Hpa*II, insensitive to methylation, was used as a positive control. The PCR probe spans the βRARE and the TATA box regions (Figure 1a). The same 7.5 kb region was previously analysed in a colon carcinoma cell line, and the size of all the possible fragments relative to the most 3′*Hpa*II site were reported (Cote' and Momparler, 1997). A representative blot is shown in Figure 1b. Genomic DNA from the ER-positive, RA-inducible cell line T47D is digested to completion, indicating that it is not methylated at any of the *Hpa*II sites. In contrast, DNA from the ER-positive, RA-inducible ZR75-1 cell line and DNA from the ER-negative, RA-resistant MDA-MB-231 cell line showed to be differentially methylated at the methylation-sensitive sites. (Figure 1b). Using methylation-specific PCR (MSP), we further analysed a 616 bp long *RARβ2* region from nucleotide 481 to nucleotide 1096 (Shen *et al*., 1991) in all the cell lines. MSP entails the modification of genomic DNA by sodium bisulfite that converts all unmethylated, but not methylated, cytosine to uracil (Herman *et al*., 1996). The distribution of CpGs expected after Na bisulfite modification and the four MSP primers (1–4) is reported in Figure 2a. The genomic DNAs from four breast cancer cell lines ZR751, MCF7, MDA-MB-231, MDA-MB-468 showed partial to complete methylation of the promoter region (Figure 2b). The human mammary epithelial cell (HMEC) strain 48R, expressing *RARβ* and three breast cancer cell lines, the *RARβ*-positive Hs578t and the RA-inducible MDA-MB-435 and T47D, revealed only the (U) unmethylated PCR products (Figure 2b).

These results indicate that hypermethylation of the *RARβ2* promoter occurs in breast cancer cell lines irrespective of the ER status, and can be detected in both RA-inducible, and RA-resistant breast cancer cells.

RARβ2 *is unmethylated in both mortal and immortalized HMEC, but is methylated in primary breast tumors*

Next, we asked whether hypermethylation of *RARβ2* promoter in cell lines has correlates in clinical breast cancer. As a normal control we analysed the HMEC mortal strains (48R, 172R), that are the closest representation of normal mammary epithelial cells available. We also analysed two immortal mammary epithelial strains (184A1 and 184B5). The DNA of these strains was found to be unmethylated (Figure 2c). Consequently, methylation of *RARβ2* may be an event in the progression of breast cancer, following immortalization. Genomic DNAs from three paraffinated samples of breast tumors, two ER-positive (T1, T2) and one ER-negative (T3), estimated to contain more than 90% tumor cells, were analysed with all MSP primer pairs, and shown to be partially methylated (Figure 2d). Both microdissected breast stroma, and microdissected normal epithelial cells were found unmethylated at *RARβ2* (our unpublished observations), making it very likely that the U products in the tumor samples were amplified either from residual normal epithelial cells, or stromal cells mixed to tumor cells. DNAs from matching histologically tumor free lymph node samples (N1–N3), were similarly analysed and produced only the unmethylated PCR products (Figure 2d). The DNA of additional 21 tumors was performed using two sets of primer pairs (U3/M3 and U4/M4). Fifteen (7 ER-positive and 8 ER-negative) of the 24 tumors presented methylation at the *RARβ2* promoter. With the same primer sets hypermethylation at *RARβ2* was detected in the DNA of ten out of 39 primary breast tumors collected, and analysed independently, at the Johns Hopkins University.

The overall data indicate that hypermethylation at *RARβ2* promoter occurs in approximately one third of primary breast tumors, and that the *RARβ2* methylation state is independent of the ER status of the tumor.

5-Aza-CdR induces partial demethylation at the RARβ2 *CpG island and reactivation of* RARβ *gene expression*

In order to determine whether DNA methylation is affecting, at least in part, *RARβ* gene expression, we treated all the cell lines showing methylation at the *RARβ2* promoter with the DNA methyltransferase inhibitor, 5-Aza-CdR. Treatment of cells with either

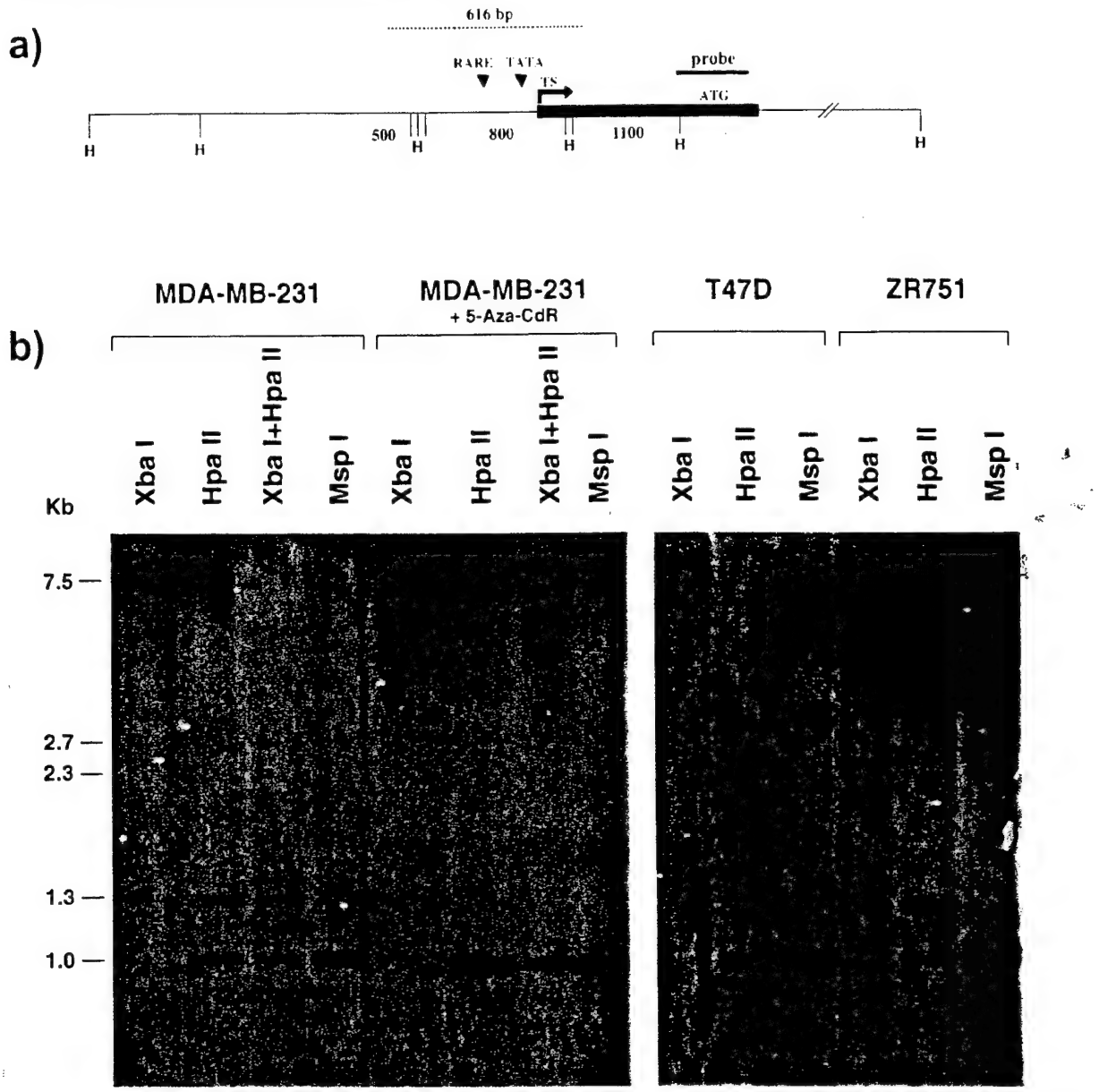

Figure 1 Methylation sensitive Southern blotting of the *RARβ2* promoter. (**a**) Genomic map of the *RARβ2* promoter-exon 5 region indicating the position of *Hpa*II sites (H) relative to βRARE, TATA, transcription start site (TS) and the ATG. (**b**) Southern analysis of : T47D and ZR571 DNAs digested with *Xba*I, *Hpa*II (right) and *Msp*I and MDA-MB-231 cells before, and after treatment with 0.8 μM 5-Aza-CdR for 3 days (left)

0.4 or 0.8 μM 5-Aza-CdR for 3 days, led to partial demethylation of the CpG rich *RARβ2* region. This was evident both by Southern analysis in the MDA-MB-231 cell line (Figure 1b, left panel), and by MSP in all cell lines (Figure 3). Moreover, 5-Aza-CdR treatment resulted in reactivation of gene expression both in RA-inducible MCF7 and ZR75-1, and RA-resistant MDA-MB-231 and MDA-MB-468 cells (Figure 4b). We asked whether reactivation of *RARβ* expression by 5-Aza-CdR A-resistant cells could be enhanced by RA. By using non-quantitative RT–PCR, we could not appreciate a difference in the level of *RARβ* transcription in MDA-MB-231 cells treated with 0.4 μM 5-Aza-CdR alone, or in combination, with 1 μM RA (Figure 4c). In this experiment, 5-Aza-CdR alone, or in combination with RA, produced 63 and 96% growth inhibition respectively. In the same experiment, treatment with 1 μM RA alone produced a negligible effect on growth inhibition (<2%). A synergistic effect of the two drugs on cancer cells was previously reported (Cote' and Momparler, 1997; Bovenzi *et al*., 1999).

These data indicate that DNA methylation is, at least, one factor influencing the downregulation/loss of *RARβ* transcription in breast cancer cell lines with a methylated *RARβ2* promoter. Cells treated with 5-Aza-CdR alone, or in combination with RA, showed re-expression of *RARβ*, which may have contributed, along with the toxic 5-Aza-CdR, to the observed growth inhibition.

The HDAC inhibitor TSA can reactivate RARβ *expression in RA-resistant cells; demethylation of the* RARβ2 *promoter is not an absolute requirement for* RARβ *reactivation*

The chromatin status at a given locus can be dynamically influenced by the degree of acetylation/

deacetylation due to HAT/HDAC activities. Absence of *RARβ* regulatory factors, like RARα, as well as DNA-methylation, can contribute to pattern chromatin modifications at *RARβ* promoter in RA-resistant cell lines. One of these cell lines, MDA-MB-231, lacks RA-inducible *RARα* activity (Shao *et al*., 1994) and displays a *RARβ2* methylated promoter. We decided to probe indirectly whether the level of HDAC at *RARβ2* can influence *RARβ* expression, by testing the effect of TSA, a HDAC inhibitor on MDA-MB-231 cells (Yoshida *et al*., 1995). Cells were treated for 2 days, in the presence or absence of 100 ng/ml TSA alone, or in combination, with 1 μM RA. By using RT–PCR, it was clear that, unlike cells treated with RA alone, cells treated with a combination of RA and TSA re-expressed *RARβ* mRNA (Figure 4d). Under the same experimental conditions, 100 ng/ml TSA alone, or in combination with 1 μM RA, produced 77

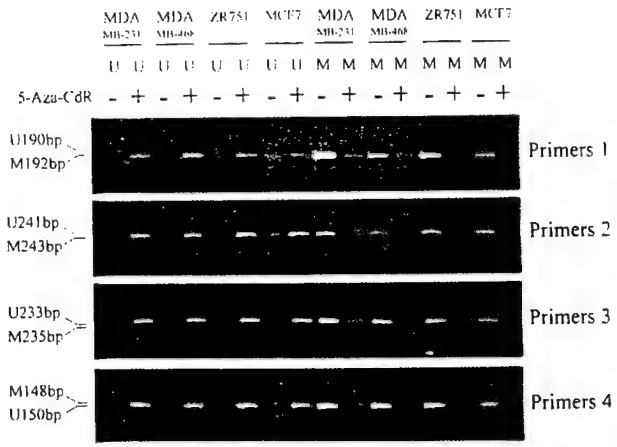

Figure 3 Treatment with 5-Aza-CdR induce partial de-methylation. MSP analysis of DNA of four breast cancer cell lines before and after treatment for 3 days with 0.8 μM 5-Aza-CdR

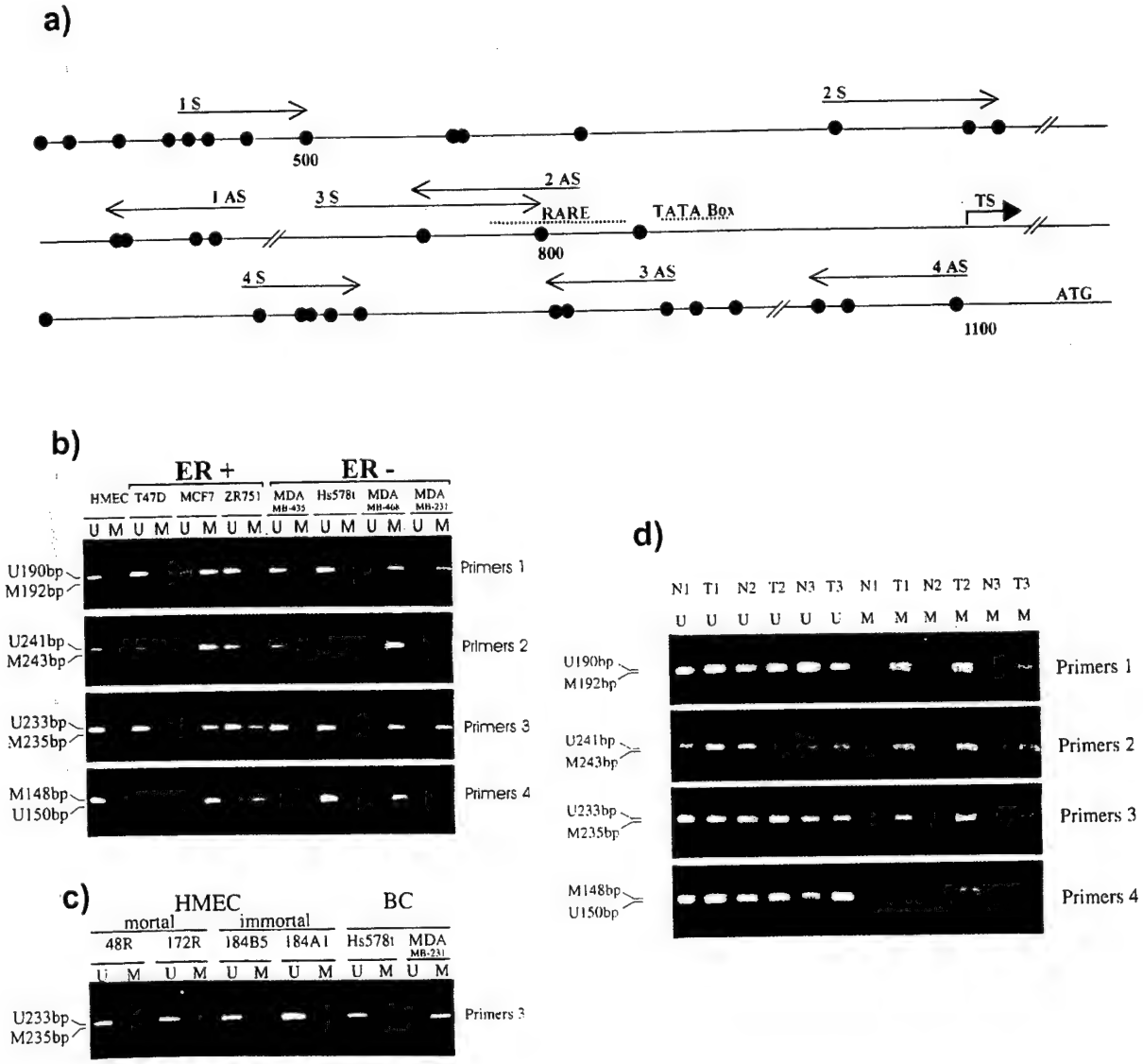

Figure 2 MSP analysis of DNA from cell lines and primary tumors. (**a**) Distribution of the methylated CpGs (filled circles) in the *RARβ2* promoter region spanning nt 498 to nt 1096 and position of the MSP primers. (**b**) MSP analysis of a panel of breast carcinoma cell lines. U and M products amplified with the four sets of MSP primers in ER-positive and -negative cell lines and the mortal HMEC (48R) strain. (**c**) MSP analysis of two mortal (48R and 172R) and two immortal (184A1 and 184B5) HMEC strains. (**d**) MSP analysis of three breast tumors (T1–T3) and matching tumor cell free lymph nodes (N1–N3)

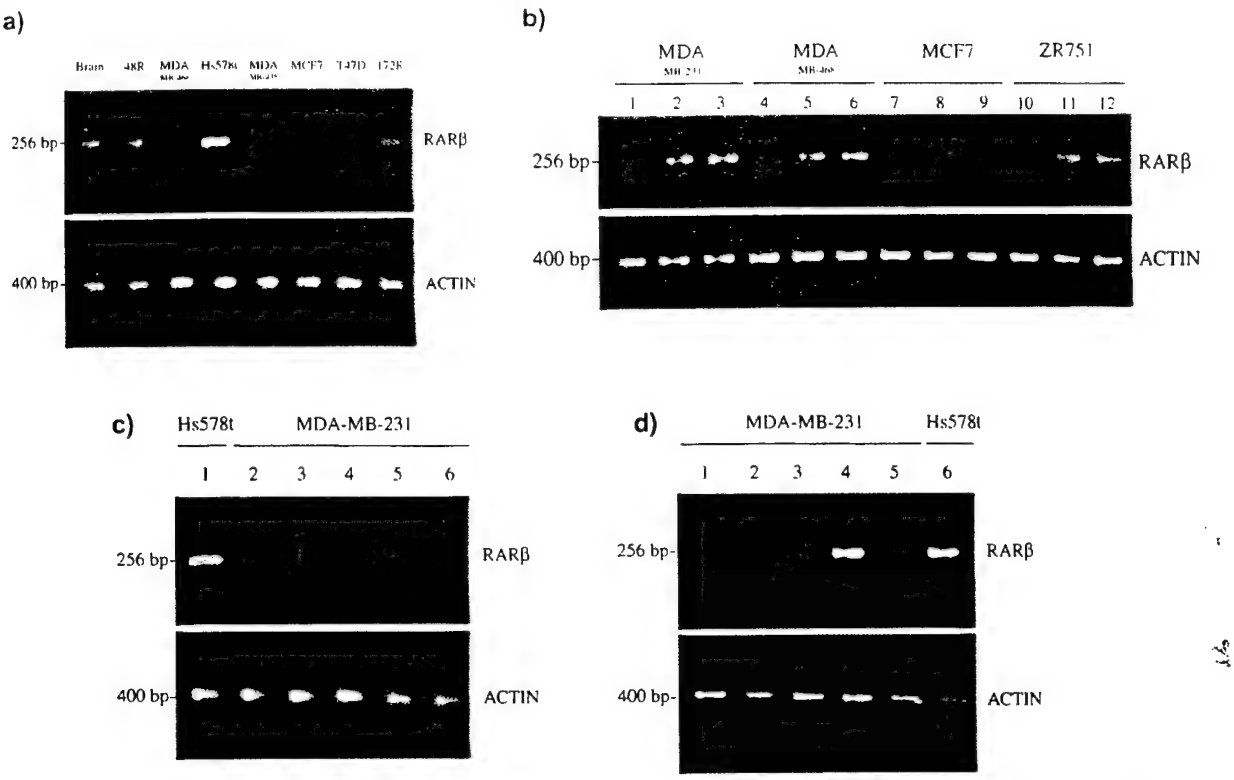

Figure 4 Treatment with 5-Aza-CdR and TSA triggers re-expression of *RARβ*. (**a**) RT–PCR of mortal HMEC strains 48R and 172R and breast cancer cell lines. Brain RNA was used as a control. (**b**) RT–PCR of breast cancer cell lines treated for 3 days with 0.4 μM (lanes 2, 5, 8 and 11) and 0.8 μM (lanes 3, 6, 9 and 12) 5-Aza-CdR and untreated cells (lanes 1, 4, 7 and 10). (**c**) RT–PCR of MDA-MB-231 cells untreated (lane 2) in comparison with cells treated for 3 days with 1 μM RA (lane 3), 0.4 μM 5-Aza-CdR (lane 4), 0.4 μM 5-Aza-CdR + 1 μM RA (lane 5). Hs578t used as positive control (lane 6). (**d**) RT–PCR of MDA-MB-231 cells untreated (lane 1), in comparison with cells treated for 48 h with 1 μM RA (lane 2), 100 ng/ml TSA (lane 3), 100 ng/ml TSA + 1 μM RA (lane 4); solvent (lane 5)

and 92% growth inhibition, respectively. Treatment with 1 μM RA alone did not affect significantly growth inhibition (<2%). By MSP analysis, we could assess that *RARβ* expression was restored in the presence of a methylated *RARβ2* promoter. The MSP profile obtained with primer set 3, spanning the βRARE region is reported in Figure 5. This finding indirectly shows that global alterations of HDAC activity, generated by TSA in MDA-MB-231 cells, involved *RARβ2* resulting in RA-induced *RARβ* expression. Further, demethylation at *RARβ2* did not seem to be an absolute requirement for *RARβ* gene expression in MDA-MB-231 cells. Noteworthy, persistence of methylation at *RARβ2* was observed also in MCF7 cells where *RARβ* transcription could be restored in the presence of RA (data not shown). Growth inhibition was observed in cells treated with TSA alone, or in combination, with RA. Very likely, *RARβ* along with TSA, a drug known to induce growth inhibition (Yoshida *et al*., 1995), contributed to the massive growth inhibitory effect that we observed.

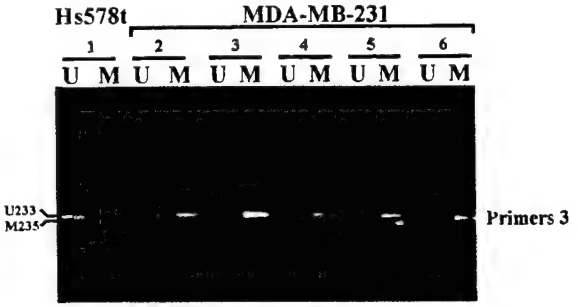

Figure 5 DNA methylation in *RARβ2* promoter of MDA-MB-231 cells expressing RA-induced *RARβ* after TSA treatment. MSP analysis of MDA-MB-231 cells using primers 3 shows that there is no demethylation of the βRARE containing region in cells treated with 1 μM RA (lane 3), 100 ng/ml TSA (lane 4), 100 ng/ml TSA + 1 μM RA (lane 5); in comparison with untreated cells (lane 2), or cells grown in the presence of solvent (lane 6). MSP of Hs578t was used as a control (lane 1)

Discussion

RARβ2 *promoter is methylated in breast cancer*

In this study, we show evidence that, in breast cancer cells, *RARβ2* promoter undergoes DNA hypermethylation, an epigenetic change known to induce chromatin modifications and influence gene expression (Razin, 1998; Ng and Bird, 1999; Jones and Wolffe, 1999). We detected methylation of the *RARβ2* promoter region, both in breast carcinoma cell lines, and a significant proportion of primary breast tumors. *RARβ2* methylation status did not correlate with the ER status of breast cancer cells and was observed both in *in situ* lesions and invasive tumors (our unpublished observations).

It is not clear when epigenetic changes occur during breast cancer progression. However, methylation of the promoter was not detected in both mortal, and

immortal human mammary epithelial cell (HMEC) strains, as well as in normal microdissected breast epithelial cells (our unpublished observations). These results suggest that aberrant methylation of the *RARβ2* CpG island may be a later event following immortalization. Treatment of breast cancer cells presenting with a methylated *RARβ2*, with the demethylating agent 5-Aza-CdR, induced partial DNA demethylation and restored *RARβ* gene expression. This evidence clearly indicates that DNA methylation is at least a component contributing to *RARβ* downregulation/loss.

RARβ2 *methylation state and RA-inducibility*

The correlation between *RARβ2* methylation and RA-inducibility in different breast cancer cell lines, indicates that DNA methylation is not the only factor influencing *RARβ* silencing. Survey of different breast cancer cell lines shows that *RARβ* is downregulated, but can be reinduced by RA both in MDA-MB-435 and T47D cells, with unmethylated *RARβ2* promoter and in MCF7 and ZR751 cells, with a methylated promoter. In contrast, in MDA-MB-231 and MDA-MB-468 cell lines the methylated *RARβ2* promoter is indifferent to RA treatment. Apparently, different degrees of repression can affect *RARβ2* promoter, and only in some cases, the ligand is sufficient to alleviate methyl-directed repression. Extinction of *RARβ* transcription must be determined by a stable repressive state in the chromatin structure determined by more than one mechanism, including DNA methylation.

DNA-methylation might be secondary to RARβ2 *promoter inactivity*

We hypothesize that low intracellular levels of RA in breast cancer cells may induce chromatin structure alterations at *RARβ2*, similar to the ones observed in the P19 embryonal carcinoma cell line (Bhattacharyya *et al*., 1997). Although the mechanism of chromatin structure alterations are not fully understood, current evidence indicates that local histone acetylation is a crucial factor (Razin, 1998). An altered chromatin environment may predispose to DNA methylation, a condition that might further affect histone deacetylation at *RARβ2* (Razin, 1998; Ng and Bird, 1999; Jones and Wolffe, 1999). The first to propose that gene inactivity 'invites' *de novo* methylation was Bird (1986). The hypothesis was further refined, after the discovery of the mechanistic link between DNA methylation and chromatin conformation mediated by the MeCP2/Sin3A/HDAC corepressor complex (Nan *et al*., 1998; Wade *et al*., 1998). According to the revisited hypothesis, Ng and Bird (1999) propose that: 'DNA methyltransferase – either independently or assisted by accessory proteins – may be capable of reading the histone acetylation pattern on the chromatin and its *de novo* methyltransferase activity can respond differentially to different states of chromatin modification. In this case, deacetylated chromatin would provoke *de novo* methylation. This self-reinforcing mechanism, supported by DNA methylation and histone deacetylation, could provide a stable state of inactive chromatin, unless overcome by other mechanisms'.

RARβ2 promoter in breast cancer might provide an ideal system to test this hypothesis, given the heterogeneous correlation between its methylation state and RA-inducibility in different breast cancer cells. Unmethylated, RA-inducible *RARβ2* promoters are expected to be associated with, either an active chromatin state, or a mild repressive state. A methylated *RARβ2* promoter is expected to be associated with a more repressive chromatin environment. As a consequence, transcription from a methylated promoter should be possible, either by recruiting consistent HAT activity, or by inhibiting excessive HDAC activity. These speculations are so far supported by compelling circumstantial evidence. Notably, RA can induce both RARα and RARβ in MCF7 cells from a *RARβ2* methylated promoter (Shao *et al*., 1994; Shang *et al*., 1999; our unpublished observations). This suggests that RA may trigger recruitment of HAT activity at *RARβ2*, sufficient to override methylation-related chromatin constraints. On the contrary, in the MDA-MB-231 cells we saw that RA-induced *RARβ* transcription is possible after treatment with TSA, a HDAC inhibitor, already known to induce chromatin alterations at *RARβ2* promoter in P19 cells (Minucci *et al*., 1997). Analysis of the DNaseI sensitivity pattern, in and around *RARβ2*, as well as the assessment of *RARβ2* histone acetylation state (Keshet *et al*., 1986; Hebbes *et al*., 1994; Eden *et al*., 1998), in both MCF7 and MDA-MB-231 cells will give us an idea of the relation between chromatin environments and RARβ transcription. Moreover, these studies are expected to shed light on the relation of histone acetylation and methylation of the *RARβ2* promoter. This issue is of particular interest since it is not yet completely clear whether DNA demethylation is indeed always required to restore transcription from genes with fully methylated promoters (Cameron *et al*., 1999; Ferguson *et al*., 1998; Razin, 1998; Ng and Bird, 1999).

In conclusion, we provide evidence that DNA-methylation at *RARβ2* promoter in breast cancer cells is affecting, at least in part, *RARβ* transcription. We argue that DNA-methylation is secondary to the inactive state at *RARβ2* promoter and may contribute to create a stable repressive *RARβ2* environment and extinction of *RARβ* transcription. Further understanding of epigenetic changes and chromatin alteration at *RARβ2* may have preventive and therapeutic implications. Changes altering *RARβ2* chromatin structure and *RARβ* transcription in breast cancer might be prevented in the presence of supraphysiological levels of RA (Minna and Mangeldorf, 1997). Knowledge of *RARβ2*-methylation state of primary breast cancers might be useful to identify tumors that are more likely to respond to RA-therapy. Finally, the possibility to re-induce *RARβ* activity in RA-resistant breast cancer cells, using both TSA and RA, a combination proven to be effective for treating leukemia (Grignani *et al*., 1998; Guidez *et al*., 1998; He *et al*., 1998; Lin *et al*., 1998; Warrell *et al*., 1998), might have therapeutic implications also in the treatment of RA-resistant breast tumors.

Materials and methods

Cell cultures

Human epithelial mammary cells (HEMC) from reduction mammoplasty including three mortal strains, 184, 48R and

172R, and two immortal strains, 184A1 and 184B5, were obtained and cultured according to the protocols designed by Dr Martha Stampfer (see the HMEC Homepage, http://www.lbl.gov/~mrgs/index.htlm) using Clonetics (Walkersville, MD, USA) reagents.

Human breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium (GIBCO) (Hs578t, MCF-7, MDA-MB-231 and T47D) or IMEM medium (Biofluids) (MDA-MB-435, MDA-MB-468, ZR751) with 5% fetal calf serum (FCS). For drug treatments, exponentially growing cells were seeded in 10 cm^2 plates at a density of 3×10^5 cells/plate or in 6-well plates at 1×10^5 cells/well. Cells were allowed to attach overnight before the addition of the appropriate concentration of 5-Aza-2′ deoxycytidine (5-Aza-CdR) (Sigma), Trichostatin A (TSA) (Sigma) or RA (Sigma). When reduction of retinoids was required, cells were treated in either medium with 0.5% FCS or charcoal-dextran stripped FCS (Hyclone). At the indicated time points, both attached and detached cells were harvested, counted with Trypan Blue (Life Technologies) and processed for DNA or RNA extraction. 5-Aza-CdR was dissolved in 0.45% NaCl containing 10 mM sodium phosphate (pH 6.8). Trichostatin A and all-trans-retinoic acid (RA) (Sigma) were reconstituted in absolute ethanol (solvent). The growth inhibition (%) was calculated as: $(1\text{-NT/NC}) \times 100$, where NT is the number of treated cells and NC is the number of control cells.

Tissue samples

Normal and tumor tissues were collected from existing tumor banks (Instituto per lo Studio e la Cura dei Tumori, Milan; the Cancer Center, Rotterdam, the Johns Hopkins Breast Cancer Program, Baltimore, MD, USA). All tumor samples were obtained from excess clinical specimens and institutional guidelines for the acquisition and maintenance of such specimens were followed.

DNA and RNA extraction

Extraction of DNA and RNA from breast cancer cell lines was performed by using DNAzol and Trizol respectively (Life Technologies) according to the manufacturer's instructions. Genomic DNA was further treated with 500 μg/ml proteinase K at 55°C, extracted with phenol-chloroform-isoamylic alcohol (24 : 24 : 1) (CIA) and ethanol precipitated. Extraction of DNA from paraffinated breast cancer and lymph node tissues was essentially performed as previously described (Formantici *et al*., 1999). One to three consecutive sections estimated to contain at least 90% tumor cells were incubated at 58°C overnight in 200 μl of extraction buffer (50 mM KCl, 10 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, and the solution was heated at 95°C for 15 min to inactivate the proteinase K and then centrifuged at 6000 r.p.m. The DNA in the supernatant was used for analysis.

Southern blotting

Genomic DNA (7 μg) was digested overnight with 15 U/μg of *Xba*I, *Hpa*II and *Msp*I enzymes, electrophoresis on a 0.8% agarose gel and transferred to Hybond-N filter. A 227 bp probe was amplified using the sense 5′-AGA GTT TGA TGG AGT TGG GTG GAG-3′ and antisense 5′-CAT TCG GTT TGG GTC AAT CCA CTG-3′ primers, gel purified and labeled with ^{32}P-dCTP using the Megaprime DNA labeling system (Amersham). After hybridization the filters were washed and exposed to X-ray film at −80°C for autoradiography.

Methylation specific PCR (MSP)

Bisulfite modification of genomic DNA was essentially performed as described by Herman *et al*. (1996). Modified DNA was used immediately or stored in aliquots at −20°C. The PCR mixture contained 1 × PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris (pH 8.7), 1.5 mM $MgCl_2$), dNTPs (each at 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA (50 ng) or unmodified DNA (50 ng). Reactions were hot started at 95°C before the addition of 2.5 U of Taq polymerase (Qiagen). Amplification was carried out in a Thermal Cycler 480 Perkin Elmer for 30 cycles (1 min at 94°C, 1 min at the annealing temperature (at) selected for each primer pair, 1 min at 72°C), followed by 4 min at 72°C. Twelve μl of the PCR reaction were electrophoresed onto 1.5% agarose gels, stained with ethidium bromide and visualized under UV. Two primer pairs, W3 sense 5′-CAGCCCGGGTAGGGTTCACC-3′, W3 antisense 5′-CCGGATCCTACCCCGACGG-3′, and W4 sense 5′-CCGAGAACGCGAGCGATCC-3′ and W4 antisense 5′-GGCCAATCCAGCCGGGGCG-3′, were designed on the human *RARβ2* sequence (Shen *et al*., 1991) and used to control the Na bisulfite modification. The primer pairs selected to detect the unmethylated DNA were as follows: U1 sense 5′-GTG GGT GTA GGT GGA ATA TT-3′ and U1 antisense 5′-AAC AAA CAC ACA AAC CAA CA-3′ (at 55°C); U2 sense 5′-TGT GAG TTA GGA GTA GTG TTT T-3′ and U2 antisense 5′-TTC AAT AAA CCC TAC CCA-3′ (at 49°C); U3 sense 5′-TTA GTA GTT TGG GTA GGG TTT ATT-3′ and U3 antisense 5′-CCA AAT CCT ACC CCA ACA-3′ (at 55°C); U4 sense 5′-GAT GTT GAG AAT GTG AGT GAT TT-3′ and U4 antisense 5′-AAC CAA TCC AAC CAA AAC A-3′ (at 55°C); The sequences of the primers to detect the methylated DNA were: M1 sense 5′-AGC GGG CGT AGG CGG AAT ATC-3′ and M1 antisense 5′-CAA CGA ACG CAC AAA CCG ACG-3′ (at 63°C); M2 sense 5′-CGT GAG TTA GGA GTA GCG TTT C-3′ and M2 antisense 5′-CTT TCG ATA AAC CCT ACC CG-3′ (at 57°C); M3 sense 5′-GGT TAG TAG TTC GGG TAG GGT TTA TC-3′ and M3 antisense 5′-CCG AAT CCT ACC CCG ACG-3′ (at 64°C); M4 sense 5′-GTC GAG AAC GCG AGC GAT TC-3′ and M4 antisense 5′-CGA CCA ATC CAA CCG AAA CG-3′ (at 64°C).

The distrubution of the CpG methylated sites and the position of the primers is reported in Figure 2. M and U primers were designed in the same regions, with one or two nucleotide differences to meet annealing requirements. Fragment M3 (position 773–1007) contains the βRARE (792–808) and the transcription start site (position 844); fragment M4 (position 949–1096) contains an Sp1 element (position 1074–1081).

RT–PCR

The exon 5 (sense primer 5′-GAC TGT ATG GAT GTT CTG TCA G-3′) and exon 6 (antisense primer 5′-ATT TGT CCT GGC AGA CGA AGC A-3′) were designed on the basis of published RARβ2 transcript (de The' *et al*., 1990; van der Leede *et al*., 1992) and used to amplify 50 ng of DNase treated total RNA using the Superscript One-Step RT–PCR System (Life Technologies). RT–PCR with actin primers (sense primer 5′-ACC ATG GAT GAT GAT ATC G-3′ and antisense primer 5′-ACA TGG CTG GGG TGT TGA AG-3′ was used as an internal RNA control.

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Promoter Methylation and Silencing of the Retinoic Acid Receptor-β Gene in Lung Carcinomas

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Background: **Retinoic acid plays an important role in lung development and differentiation, acting primarily via nuclear receptors encoded by the retinoic acid receptor-β (RARβ) gene. Because receptor isoforms RARβ2 and RARβ4 are repressed in human lung cancers, we investigated whether methylation of their promoter, P2, might lead to silencing of the RARβ gene in human lung tumors and cell lines.** ***Methods:*** **Methylation of the P2 promoter from small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) cell lines and tumor samples was analyzed by the methylation-specific polymerase chain reaction (PCR). Expression of RARβ2 and RARβ4 was analyzed by reverse transcription–PCR. Loss of heterozygosity (LOH) was analyzed by PCR amplification followed by electrophoretic separation of PCR products. Statistical differences were analyzed by Fisher's exact test with continuity correction.** ***Results:*** **The P2 promoter was methylated in 72% (63 of 87) of SCLC and in 41% (52 of 127) of NSCLC tumors and cell lines, and the difference was statistically significant (two-sided $P<.001$). By contrast, in 57 of 58 control samples, we observed only the unmethylated form of the gene. Four tumor cell lines with unmethylated promoter regions expressed both RARβ2 and RARβ4. Four tumor lines with methylated promoter regions lacked expression of these isoforms, but demethylation by exposure to 5-aza-2′-deoxycytidine restored their expression. LOH at chromosome 3p24 was observed in 100% (13 of 13) of SCLC lines and 67% (12 of 18) of NSCLC cell lines, and the difference was statistically significant (two-sided $P = .028$).** ***Conclusions:*** **Methylation of the RARβ P2 promoter is one mechanism that silences RARβ2 and RARβ4 expression in many lung cancers, particularly SCLC. Chemical demethylation is a potential approach to lung cancer therapy. [J Natl Cancer Inst 2000;92:1303–7]**

High frequencies of loss of heterozygosity (LOH) at chromosomal region 3p21–3p24 occur in several tumor types, suggesting the presence of one or more tumor suppressor genes in the short arm of chromosome 3 *(1–5)*. Among the genes known to map within this frequently deleted region is the retinoic acid receptor-β (RARβ) gene.

Retinoids, analogues of vitamin A, are needed for normal lung development and differentiation *(6,7)*. They can reverse preneoplastic lesions and prevent second primary tumors of the upper aerodigestive tract *(8)*. These effects are mediated via nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which stimulate transcription factors in response to binding of a retinoic acid ligand. Each receptor group includes three subtypes (α, β, and γ). Receptors of the RAR family are differentially expressed during development and in adult life, and there is strong evidence that RARβ plays a central role in growth regulation of epithelial cells and in tumorigenesis *(9–11)*.

The human RARβ gene generates multiple isoforms by use of promoters P1 and P2 and alternative splicing *(12,13)*. P1 directs the transcription of isoform RARβ1, whereas P2 promotes the transcription of isoforms RARβ2 and RARβ4 *(14)*. Isoform RARβ3 is expressed from the P1 promoter in mice, but it is absent in humans. These isoforms have been shown to vary in their ability to trans-activate retinoic acid-responsive promoters. It is thought that the receptors, through diversity in structure and patterns of expression, are able to control different subsets of retinoic acid-responsive genes to achieve the multiple effects of retinoic acid.

Repression of RARβ occurs in non-small-cell lung cancers (NSCLCs) *(5,15,16)* as well as in other human malignancies. Human lung cancers show reduced expression of RARβ2 and RARβ4 messenger RNAs (mRNAs) and protein *(5,15)*, and they exhibit resistance to retinoic acid *(17,18)*. RARβ2 plays an important role in suppression of murine lung tumorigenesis *(19)* and inhibits the growth of human lung cancer cells *in vitro (18)*. These observations suggest that loss of expression of RARβ, especially that of the RARβ2 isoform, may be associated with lung carcinogenesis. In contrast, RARβ1 is expressed in small-cell lung cancer (SCLC) cell lines *(20)*.

Analysis of lung cancer cell lines covering the entire open reading frame and the sequences of the retinoic acid response elements failed to show any mutations *(17)*. Aberrant methylation of CpG islands was identified as an epigenetic mechanism for the transcriptional silencing (inactivation) of tumor suppressor genes *(21,22)*. We investigated the role of aberrant methyl-

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See "Notes" following "References."

ation of the RARβ gene promoter P2 in human lung cancers and cell lines.

Materials and Methods

Cell Lines and Tumor Samples

All human lung cancer cell lines (66 SCLC lines and 78 NSCLC lines) and B-lymphoblastoid lines (n = 31) were established by us *(23)*. The cells were grown in RPMI-1640 medium (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) supplemented with 5% fetal bovine serum and were incubated in 5% CO_2. Samples of tissue from 49 surgically resected primary NSCLC tumors, along with 24 samples of nonmalignant lung tissue from the same patients, were obtained from the Tumor and Tissue Repository at the Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas. Formalin-fixed, paraffin-embedded sections of 21 SCLC tumors were obtained from The University of Texas M. D. Anderson Cancer Center, Houston, or from Canisius Wilhelmina Hospital, Nijmegen, The Netherlands. Archival paraffin sections of tumor-negative lymph nodes from 12 of the NSCLC patients were utilized as controls for the paraffin-embedded tumors. Epithelial cells from buccal swabs (n = 12) and peripheral blood lymphocytes (n = 10) were collected from 22 healthy volunteers and served as negative controls for the frozen tissues. Appropriate Institutional Review Board permission was obtained from all participating centers, and written informed consent was obtained from all volunteers before usage of prospectively collected fresh samples. Institutional guidelines do not require written permission for the use of anonymous archival paraffin-embedded specimens.

Genomic DNA was obtained from cell lines, primary tumors, and nonmalignant cells by digestion with 200 μg/mL proteinase K (Life Technologies, Inc.) for 1 day at 37 °C, followed by two extractions with phenol–chloroform (1 : 1) *(24)*. DNA was extracted from paraffin sections after precise laser-capture microdissection of the tumor cells or lymph nodes, as described previously *(25)*.

Methylation-Specific Polymerase Chain Reaction

The methylation-specific polymerase chain reaction (PCR) employs an initial bisulfite reaction to modify the DNA. As a result, all unmethylated cytosines are deaminated and converted to uracils, while 5-methylcytosines remain unaltered. Thus, after bisulfite treatment, alleles that were originally methylated have DNA sequences different from those of their corresponding unmethylated alleles, and these differences can be used to design PCR primers that are specific for methylated or unmethylated alleles.

DNA was treated with sodium bisulfite as described previously *(26)*. Briefly, 1 μg of DNA was denatured by incubation with 0.2 *M* NaOH for 10 minutes at 37 °C. Aliquots of 10 m*M* hydroquinone (30 μL) (Sigma Chemical Co., St. Louis, MO) and 3 *M* sodium bisulfite (pH 5.0, 520 μL) (Sigma Chemical Co.) were added, and the solution was incubated at 50 °C for 16 hours. Treated DNA was purified by use of a Wizard DNA Purification System (Promega Corp., Madison, WI). Modified DNA was stored at −70 °C until used.

Amplification of bisulfite-modified DNA for RARβ gene promoter P2 was performed by PCR as described by Côté et al. *(27)* with primers that were specific for either methylated or unmethylated RARβ sequences. Primers used to amplify the methylated RARβ gene were 5′-TCGAGAACGCGAGCGATTCG-3′ (sense) and 5′-GACCAATCCAACCGAAACGA-3′ (antisense). Primers used to amplify the unmethylated RARβ gene were 5′-TTGAGAATGTGAGTGATTTGA-3′ (sense) and 5′-AACCAATCCAACCAAAACAA-3′ (antisense). Normal lymphocyte DNA was treated with *Sss*I DNA methyltransferase (New England Biolabs, Inc., Beverly, MA), subjected to bisulfite modification, and used as a positive-control DNA for each PCR reaction *(28)*. Negative control samples without DNA were included for each set of PCR. PCR of DNA from nonmalignant tissues and samples from healthy volunteers served as negative (unmethylated) controls. PCR products were analyzed on 2% agarose gels containing ethidium bromide (Life Technologies, Inc.). Conversion of all unmethylated Cs to Ts was confirmed by sequencing eight individual PCR products.

Reverse Transcription-PCR Analysis

Four tumor cell lines in which RARβ P2 promoter had been identified as being methylated were incubated in culture medium with and without 5-aza-2′-deoxycytidine (Aza-CdR) at a concentration of 2 μg/mL for 6 days, with medium changes on days 1, 3, and 5 *(29)*. Cells were harvested at the end of the 6th day for extraction of mRNA with a polyadenylic acid tail [poly(A) RNA]. Reverse transcription (RT) was performed on poly(A) RNA with the SuperScript II First-Strand Synthesis System (Life Technologies, Inc.), with the use of RARβ2-gene-specific reverse primer *(29)*. One microliter of the RT reaction mixture was used as template for PCR with primer set 1 *(29)* to produce a 256-base-pair (bp) RARβ2 gene product. A separate PCR was performed on the same RT product by use of primer set 2, which consists of forward primer 110-FP *(30)* and the above-described RT reverse primer, to amplify RARβ2 (623-bp product) and RARβ4 (264-bp product) simultaneously. One-step RT–PCR (Life Technologies, Inc.) was performed with primers for glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene (sense primer: 5′-ACAGTCCATGCCATCACTGCC-3′; antisense primer: 5′-GCCTGCTCCACCACCTTCTTG-3′), to confirm the integrities of the poly(A) RNAs. PCR products were analyzed on 2% agarose gels.

Analysis of LOH

Fourteen polymorphic microsatellite markers (*see* Table 2) that are located in chromosome region 3p24 and flank the RARβ gene *(31)* were selected for LOH analysis. DNA from 13 SCLC and 18 NSCLC tumor cell lines and their corresponding B-lymphoblastoid lines (constitutional DNA) were analyzed as described previously *(23)*. Briefly, 20 ng of genomic DNA was amplified by PCR in the presence of [α-^{32}P]cytidine 5′-triphosphate by use of the microsatellite markers. The PCR products were separated by electrophoresis in 6% polyacrylamide gels containing 7 *M* urea and were visualized by autoradiography. Markers that identified two bands of different size but of similar intensity in the lane having normal DNA were termed "informative" (i.e., heterozygous). Markers that gave only a single major band in the normal DNA lane were termed "noninformative." LOH was defined as a loss of a band corresponding to an allele present in informative cases.

Statistical Analysis

Statistical differences between groups were examined by use of Fisher's exact test with continuity correction. All *P* values are two-sided. Values of $P<.05$ were considered to be statistically significant.

Results

Methylation-specific PCR was performed on bisulfite-modified control and tumor DNA samples by simultaneous use of primers for the methylated and unmethylated forms of the RARβ gene promoter P2. As detailed in Table 1, 72% of (63 of 87) of SCLC and 41% (52 of 127) of NSCLC samples showed

Table 1. Incidence of methylation of the RARβ gene promoter P2 in lung tumors, tumor cell lines, and control tissues

Sample*	No.	No. of methylated samples (%)†
SCLC		
Paraffin-embedded tumors	21	13 (62)
Cell lines	66	50 (76)
All SCLC samples	87	63 (72)
NSCLC		
Frozen tumors	49	21 (43)
Cell lines	78	31 (40)
All NSCLC samples	127	52 (41)
Total lung cancer samples	214	115 (54)
Nonmalignant tissues		
Peripheral blood lymphocytes‡	10	0 (0)
Epithelial cells from buccal swabs‡	12	0 (0)
Paraffin-embedded tissues	12	1 (8)
Frozen lung tissues	24	0 (0)
Total nonmalignant samples	58	1 (1.7)

*SCLC = small-cell lung cancer; NSCLC = non-small-cell lung cancer.

†The differences in methylation incidences between SCLC and NSCLC cell lines were statistically significant for cell lines (two-sided $P<.001$, Fisher's exact test) and for all samples (two-sided $P<.001$) but not for tumor tissues (two-sided $P = .19$)

‡From healthy volunteers.

Table 2. Loss of heterozygosity (LOH) at chromosome locus 3p24 in small-cell lung cancer (SCLC) and in non-small-cell lung cancer (NSCLC) cell lines

Marker*	No. of LOH/No. of informative cases (% LOH)		
	SCLC (n = 13)	NSCLC (n = 18)	P†
D3S1599	7/7 (100)	3/9 (33)	**.011**
D3S3659	7/8 (88)	6/11 (55)	.18
D3S3598	7/7 (100)	7/11 (64)	.12
D3S3700	7/8 (88)	7/9 (78)	1.00
D3S1567	6/6 (100)	4/4 (100)	—
D3S1583	5/5 (100)	3/9 (33)	**.031**
D3S2336	8/9 (89)	5/17 (29)	**.011**
D3S2335	9/9 (100)	6/12 (50)	**.019**
D3S2337	9/10 (90)	6/15 (40)	**.018**
D3S1266	10/10 (100)	5/8 (63)	.069
D3S1283	8/10 (80)	7/12 (58)	.38
D3S1609	5/5 (100)	6/10 (60)	.23
D3S3547	8/8 (100)	4/8 (50)	.077
D3S3527	7/8 (88)	7/12 (58)	.33
Any	13/13 (100)	12/18 (67)	**.028**

*While their precise order is controversial, the markers are arranged, as best as we could determine, in order from telomeric (D3S1599) to centromeric (D3S3527).

†Differences in incidence of LOH between SCLC and NSCLC cell lines were analyzed by use of Fisher's exact test. All *P* values are two-sided. Statistically significant values are printed in **boldface.**

gene, RARβ4-like expression showed tissue-specific variation *(34)* and was reduced in lung tissue. These data support the hypothesis that one or more isoforms of the RARβ gene may exert tumor-suppressive effects.

Aberrant methylation of the RARβ promoter gene has been observed previously in breast and colon cancers *(29,35,36)*. Our observations demonstrate a high frequency of aberrant DNA methylation of the RARβ P2 promoter gene in lung cancers, particularly in SCLC. In the eight cell lines that we tested, there was complete concordance between aberrant methylation of the P2 promoter and silencing of both RARβ2 and RARβ4 transcripts. Furthermore, treatment with Aza-CdR restored transcript expression, indicating that methylation is one of the mechanisms responsible for loss of expression. Allelic losses at or around 3p24, the chromosomal location of RARβ, were more frequent in SCLC (100%) than in NSCLC (67%). The high frequency of LOH at 3p24, combined with the presence of only methylated sequences in most cell lines, fulfills the criteria for Knudson's two-hit hypothesis for tumor suppressor gene inactivation *(37)*. While strong circumstantial evidence exists for the role of inactivation of the RARβ gene in lung cancer pathogenesis, the possibility that other genes at 3p are responsible for or contribute to lung cancer pathogenesis must be considered.

Several genes are known to be inactivated in lung cancers by aberrant methylation *(38)*. The frequencies of aberrant methylation of the RARβ gene reported herein are among the highest for any gene described to date in lung cancers *(38)*. Ayoub et al. *(39)* have reported frequent repression of both RARβ2 and RARβ4 in the bronchial epithelium of smokers. Their finding and those of other investigators *(33,40)* suggest that alteration in RAR expression is an early event in lung cancer pathogenesis. Tumor cells may release their DNA into the circulation, which would allow detection of aberrant methylation in DNA from the sera of lung cancer patients *(38)*. Chemical reversal of methylation-related gene silencing (an epigenetic phenomenon) is a potential therapeutic approach *(41)*. Our findings indicate that aberrant methylation of the RARβ gene is a frequent abnormality in lung cancers and may have clinical applications for risk assessment, for diagnosis, and for novel therapeutic approaches.

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NOTES

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Abstract 1
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Silencing of the retinoic acid receptor beta (RAR beta) gene by hypermethylation in human breast cancer. *S. Sirchia[1], A.T. Ferguson[2], S. Sukumar[2], R. Orlandi[3], N. Sacchi[1,2].* 1) School of Medicine,University of Milan, Milan, Italy; 2) Oncology Center, John Hopkins University, Baltimore, MD; 3) Istituto Nazionale Tumori, Milan, Italy.

CpG island hypermethylation is known to be associated with gene silencing in human cancer. It has been proposed that this epigenetic event may be determined either by aberrant DNA methyltransferase (DNA-Mtase) activity or other cellular factors. Recent evidence seems to indicate that, aside from random DNA-Mtase action, additional cellular factors exist that govern aberrant methylation in breast cancer cells. By using methylation specific PCR (MSP) we have shown that methylation of the retinoic acid receptor beta (RAR beta) promoter region containing the retinoic acid responsive element (RARE) is occurring in a great proportion of breast cancer cell lines and primary breast tumors, but not in normal human mammary epithelial cells (HMEC). Methylation of RAR beta in breast cancer cell lines is associated with loss of expression of the RAR beta gene. The expression can be restored by demethylating agents. Interestingly, we have observed that aberrant methylation of RAR beta is occurring also in normal breast tissue surrounding breast cancer cells, but not in normal lymphonodes. These observations suggest that aberrant methylation of RAR beta, leading to silencing of the RAR beta gene may be an early event in breast carcinogenesis.

Abstract 2

AACR 2000

#3931 EXPRESSION OF RAR BETA PROTEIN ISOFORMS IN RETINOIC ACID (RA)-SENSITIVE AND RESISTANT BREAST CANCER CELL LINES.

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The human RAR beta gene is transcriptionally active in normal human mammary epithelial cells, but it is markedly down-regulated in a significant proportion of primary breast cancers and the majority of breast cancer cell lines. Expression of RAR beta mRNA can be up-regulated by RA in some breast cancer cell lines, with consequent growth inhibition and/or apoptosis. In contrast, breast cancer cell lines in which RAR beta transcription cannot be induced by RA are resistant to growth inhibitory effects of RA. Recent findings by Sommer et al. (PNAS, 96: 8651-8656, 1999) have indicated that breast cancer cell lines can synthesize RAR beta isoforms in the absence of detectable RAR beta transcription. For thisreason we decided to analyze the RAR beta protein isoform profile in a panel of breast carcinoma cell lines well characterized by us and others, both for their RAR beta transcripts and RA-sensitivity. Using an antibody developed against a peptide in the C region common to both RAR beta-2 and beta-4 protein isoforms and Western blot analysis, we detected a correlation between expression of RAR beta transcripts and proteins in all the breast carcinoma cell lines so far analyzed.

DNA METHYLATION AND CHROMATIN STATE OF THE RETINOIC ACID RECEPTOR β (RAR β) PROMOTER IN BREAST CANCER CELLS

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The expression of the retinoic acid receptor β, RAR β, one of the nuclear receptors that mediates retinoic acid (RA) activity is found severely downregulated or lost in breast cancer cells. We, and others, have observed that DNA methylation can affect one of the two RAR β promoters, RAR β2, in both breast cancer cell lines and tumors. Treatment of cells with a methylated promoter by means of the DNA methyltransferase inhibitor 5-Aza-2' deoxycytidine led to demethylation of the RAR β2 promoter and expression of RAR β indicating that DNA methylation is at least one factor, contributing to RAR β inactivity. Interestingly, identically methylated promoters can differentially respond to RA, suggesting that the RAR β2 promoter activity may be associated with different levels of histone acetylation of the chromatin embedding the RAR β2 promoter.. This supposition is supported by the finding that the more stable repressive RAR β2 state in RA-resistant breast cancer cell lines can be alleviated by the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), with restoration of RA-induced RARβ transcription, in the presence of a methylated RAR β2 promoter. To understand whether DNA demethylation is always required to restore transcription from methylated promoters, we report the relation of DNA methylation and histone acetylation of the RAR β2 promoter by combining methylation specific PCR (MSP) and chromatin immunoprecipitation (ChIP) assay on both RA-inducible and RA-resistant breast cancer cell lines.

Abstract 3
Cancer Genetics and Tumor Suppressor genes
Cold Spring Harbor, 2000